

## Article

# Novel Ternary Biopolymer Films Incorporating Amygdalin: FTIR, TG, and In Vitro Evaluation on Model Bacteria

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## Abstract

The development of new composite wound dressing films that can ensure a moist environment while preventing bacterial growth led this research to obtain novel ternary biopolymer films as a carrier for amygdalin. Due to their accessibility, biocompatibility, and versatility,  $\kappa$ -carrageenan, hydroxypropyl methylcellulose, and gelatin were selected as matrix components. This novel film composite was characterized by Fourier–transform Infrared (FTIR) spectroscopy, Thermogravimetric (TG) and Derivative Thermogravimetric (DTG) analysis, and was evaluated in vitro against *E. coli* and *S. aureus*. Thermogravimetric analysis showed that increasing the amygdalin content gradually shifted the  $T_{\text{onset}}$  and  $T_{\text{max}}$  values to higher temperatures, suggesting an improvement in the thermal stability of the composite matrix. In vitro results indicate that increasing the amygdalin concentration resulted in a bacteriostatic efficiency of up to 54% against *E. coli*, while exhibiting a plateau effect in bactericidal activity. In contrast, no bactericidal activity was observed against *S. aureus* cultures.

**Keywords:** amygdalin encapsulation; cellulose derivatives; wound dressings; *E. coli*; polymer-based delivery systems; FTIR; novel biomaterials; polymer composites



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## 1. Introduction

As organic materials derived from natural sources, including plants, algae, animals, and fungi, biopolymers offer a promising alternative that can fully or partially replace synthetic polymers [1,2]. Therefore, as natural compounds, they can contribute to reducing the environmental impact and dependence on fossil resources associated with synthetic polymers [3]. Their organic origin and polymeric nature imply a linear or branched spatial structure, in which the constituent monomeric units, whether saccharides, amino acids, or nucleotides, are covalently bonded [4,5]. Depending on the chemical nature of their monomer units, they can be classified as polysaccharides (such as alginate, starch, etc.), polypeptides (oxytocin [6]), polyhydroxyalkanoates (poly(3-hydroxybutyrate) (PHB) [7]), polyamides, proteins (collagen, keratin, etc.), and polylactic acids [2].

Since its first description in the early 19th century, carrageenan has found a wide range of uses, in recent years becoming a biomaterial of major importance in biopolymer-based drug delivery systems [8]. As a biopolymer of marine origin,  $\kappa$ -carrageenan is a polysaccharide, more precisely a sulfated polygalactan, which, in addition to being able to form thermoreversible gels, also exhibits antibacterial, antiviral, and anticancer properties [9]. Another biomaterial that attracts major interest in the biomedical field is gelatin, a water-soluble polypeptide obtained by partial hydrolysis of collagen, which, due to its ability to form strong and clear gels, is a good candidate for obtaining drug delivery carriers [10,11]. As a water-soluble polymer, which also exhibits solubility in organic solvents, hydroxypropyl methylcellulose is a cellulose derivative that has found a wide range of applications, including in the field of drug delivery [12].

Properties like biodegradability and biocompatibility have made these macromolecules [13] a promising foundation for developing new medical technologies, particularly as carriers for therapeutic agents [14]. Furthermore, they can be used for various medical purposes, such as suturing, cell proliferation, tissue guidance, and controlled drug delivery [15]. Having the ability to be functionalized to suit their intended use, biopolymers can be used in a wide range of forms, including fibers, hydrogels, membranes, and films [16]. Biopolymer-based drug delivery systems have been developed to simultaneously ensure both good bioavailability of the therapeutic agent and minimal side effects on the body [17]. These systems can also provide sustained and controlled drug delivery, regardless of whether the drug is hydrophobic or hydrophilic, including therapeutic agents with short in vivo half-lives [18]. Therefore, over the years, this type of delivery system has been diversified and studied in various fields, such as: nasal administration (of insulin [19]), ocular administration (oxytetracycline [20]), and pulmonary administration of chemotherapeutics (such as doxorubicin [21]), among many other examples in the literature.

Wound healing is a biological process of major importance, which presents four distinct stages: inflammation, angiogenesis, proliferation, and maturation [22]. Wound dressings based on biopolymers, especially polysaccharides, can reduce the risk of infection, promote cell growth and tissue repair, and maintain a moist healing environment, thus ensuring proper wound healing [23]. One of the most widespread forms of wound dressings are biopolymer films, either with intrinsic antibacterial properties or loaded with an antibacterial agent [24]. Bacteria are complex microorganisms capable of developing various survival strategies, such as antibiosis, enzyme secretion, volatile compound production, and biofilm formation [25]. Therefore, the search for novel antibacterial compounds remains ongoing. The integration of bioactive compounds into polymeric matrices to obtain multifunctional antimicrobial materials represents a promising strategy for healthcare applications, including protective coatings and wound management systems [26]. Das et al. [26] reported polymer-based functional textiles with significant antibacterial activity, demonstrating the potential of composite materials for preventing microbial contamination. The results showed that the polymer composites that include silicene-based heteroatom-doped CQDs (SiCQDs) had high antibacterial efficacy against two bacterial strains, Gram-negative *E. coli* and Gram-positive *B. subtilis*, with a MIC value of 0.039 mg/mL, with the possibility of enhancing the antibacterial effect by supplementing the coating cycle [26]. While single-component dressings often have limited mechanical strength and functionality, multicomponent dressings combining natural and synthetic polymers with bioactive molecules offer enhanced antibacterial, self-healing, and stimuli-responsive properties, promoting more effective wound healing [27]. In a recently published study of ours [28], we synthesized amygdalin-loaded biopolymeric films for a possible use in wound dressings and evaluated their bactericidal activity against *E. coli* and *S. aureus*. The results showed

that the chemical nature of the biopolymers in the film matrix can directly influence the bactericidal response of the synthesized film. Films containing both  $\kappa$ -carrageenan and carboxymethylcellulose show a biological activity that varies with the concentration of incorporated amygdalin, in the case of *E. coli* culture, and no activity in the case of *S. aureus* culture. As indicated in the literature, cellulose and its derivatives do not show bactericidal properties; on the contrary, they can support and even favor the multiplication of bacterial cultures [29]. In contrast, functionalization of cellulose with quaternary ammonium salts, such as tetradecyl-trimethyl-ammonium bromide, resulted in films exhibiting 100% bactericidal activity against strains of *C. albicans*, *E. coli*, and *S. aureus* [30].

As a therapeutic agent, originally used in the treatment of cancer, Amygdalin, also known as laetrile or vitamin B17, is a cyanogenic glucoside, a natural compound often found in apple, almond and apricot seeds [31,32]. In its original form, this natural compound does not exhibit toxicity; however, upon hydrolysis by glucosidase it is converted into mandelonitrile and prunasin which in turn generate hydrogen cyanide—responsible for the final toxicity of this natural compound [33]. Once released, CN ions can inactivate cytochrome c oxidase by binding to its trivalent iron, stopping oxidative phosphorylation, thus preventing ATP synthesis, which ultimately prevents intracellular respiration and leads to cell death [34]. Thus, it was reported that amygdalin in an amount of 880 mg/kg administered orally, and 25 g/kg administered intravenously represents the lethal dose (LD50) for rats, while in humans the intravenous administration of 5 g of amygdalin represents the maximum tolerance dose (MTD) [33]. Although there is no clinical evidence to support the anticancer activity of this compound, amygdalin exhibits a number of biological activities including anti-inflammatory and analgesic activity, antioxidant, anti-asthmatic and finally antibacterial activity [35]. The study of Abdel-Gawad et al. [36] shows that the antibacterial activity was evaluated using Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and disk diffusion assays, and the antifungal activity was assessed through determination of the Minimum Fungicidal Concentration (MFC). Antimicrobial investigations demonstrated that amygdalin (AMG) exhibited broad-spectrum activity against various clinically relevant pathogenic microorganisms. Overall, the available evidence suggests that AMG possesses both antimicrobial and potential anticarcinogenic properties. These findings indicate that incorporation of AMG into protective materials, such as face masks or wound dressings, may represent a promising approach for reducing bacterial and fungal contamination, including infections associated with opportunistic pathogens. However, further studies are required to validate its clinical applicability and long-term effectiveness against resistant microorganisms [36].

Hakimi et al. [37] tested the antibacterial performance of the CS-PVA/CeTA hydrogel using the agar diffusion method against *Staphylococcus aureus* and *Escherichia coli*. The zone-of-inhibition assay demonstrated clear antibacterial activity of the CS-PVA/CeTA hydrogel against both tested strains, with inhibition zones of approximately 30 mm. These findings indicate that the hydrogel possesses intrinsic antimicrobial properties. Given that post-surgical and post-therapeutic infections represent a significant clinical concern, particularly in oncological settings, the incorporation of antimicrobial materials at the treatment site may improve therapeutic outcomes. In this context, multifunctional hydrogels that combine drug delivery capacity with antibacterial activity may contribute to reducing infection risk and potentially enhancing tissue repair processes following surgery [37].

The antibacterial activity of chitosan–NiO–amygdalin hybrid nanomaterials (HNMs) was evaluated using the well diffusion method against several Gram-positive and Gram-negative bacterial strains, including *Streptococcus pyogenes*, *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Klebsiella terrigena*, and *Klebsiella planticola* [38]. Bacterial suspensions were inoculated onto Mueller–Hinton agar plates, wells were prepared on the surface, and

different concentrations of the nanomaterial (20–100 µg/mL) were applied. Following 24 h incubation, antibacterial activity was assessed by measuring inhibition zones. The results indicated that the chitosan–NiO–amygdalin HNMs exhibited concentration-dependent antibacterial activity against all tested strains. Notably, *E. coli*, *B. subtilis*, and *K. planticola* demonstrated greater susceptibility. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values further confirmed the antimicrobial efficacy of the synthesized nanomaterials. The antibacterial mechanism of these hybrid systems has been attributed to a multifactorial mode of action. Chitosan is known to interact with negatively charged bacterial cell membranes, leading to membrane destabilization and increased permeability. NiO nanoparticles may contribute through the generation of reactive oxygen species (ROS), inducing oxidative stress, while amygdalin may interfere with bacterial metabolic pathways. The combination of these mechanisms may enhance antibacterial performance compared to single-component systems [38].

The aim of this study is the exploration of an ether derivative of cellulose in view of the development of new composite wound dressing films that can ensure a moist environment while preventing bacterial growth, thus ensuring efficient wound healing. Based on previous results [28], a new tri-polymer composite film was formulated, composed of κ-carrageenan, hydroxypropyl methylcellulose, and gelatin. This novel composite, used as a carrier for amygdalin, was characterized by FTIR spectroscopy, TG and DTG analysis, and was evaluated *in vitro* against *E. coli* and *S. aureus*, bacterial strains commonly found in infected wounds [24]. Hydroxypropyl methylcellulose (HPMC) was chosen to be incorporated into the biopolymer matrix of the films based on the premise that its branched structure (through the hydroxypropyl moiety) and derivative nature could prevent bacteria from using the film matrix as a carbon source. Although cellulose derivatives do not exhibit bactericidal activity, as mentioned earlier, exploring HPMC as a component of the films could “strengthen” the matrix, thus providing amygdalin with the possibility of acting not only against *E. coli* but also against *S. aureus*, an effect that was not observed in the previous work, where carboxymethyl cellulose was used.

## 2. Materials and Methods

### 2.1. Chemicals

The polymer base of the film matrix is formed by gelatin (CAS Number 9000-70-8), hydroxypropyl methylcellulose with average  $M_n$  of 86,000 (CAS No.: 9004-65-3) both by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and κ-carrageenan with M.W. of 788.647 g/mol by Acros Organics (Geel, Belgium). As a plasticizer, glycerol (M.W. 92.10 g/mol) by CHIMREACTIV (Ion Creanga, Romania) was used. As the active substance intended to be incorporated into the films, amygdalin with CAS. No.: 29883-15-6, with purity (HPLC)  $\geq 99\%$ , by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) was used. Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), 2,3,5-triphenyltetrazolium chloride (TTC, CAS. No.:1871-22-3), and ethanol absolute (CAS No.: 64-17-5) for *in vitro* studies were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

### 2.2. Methods

- Synthesis of amygdalin-doped films

To obtain the biopolymer base for the films intended to be doped with amygdalin, the synthesis methodology from our previous study [28] was used with some modifications. The novelty of the current synthesis protocol lies in the introduction of hydroxypropyl methylcellulose as a new component and in the combination ratio of the constituent biopolymers. To prepare the film base, κ-carrageenan, gelatin, and hydroxypropyl methylcellulose are dissolved sequentially in 10 mL of distilled water (in a mass ratio of 2:1:1 *w/w*), followed

by the addition of two drops of glycerol as a plasticizer. The dissolution of the biopolymers was performed under magnetic stirring at 500 rpm as follows: first, the carrageenan was dissolved at 85 °C, then the solution was cooled to 60 °C to dissolve the gelatin. Finally, the solution was cooled to 50 °C to dissolve the cellulose derivative and stirred for 10 min to ensure homogeneity.

To obtain the amygdalin-doped films, the following procedure was followed: four stock solutions are prepared, as described above, to which the following amounts of amygdalin are added: 5, 10, 15 and 20 mg. After adding amygdalin, the samples thus obtained are left to homogenize for 10 min on a magnetic stirrer at 500 rpm, then poured into glass Petri dishes (d = 5 cm) and placed in a preheated oven at 45 °C to dry. Once dried, four amygdalin-doped samples are obtained, as follows: κAm1 (containing 5 mg of amygdalin), κAm2 (10 mg), κAm3 (15 mg) and κAm4 (20 mg).

- FTIR analysis

The samples were analyzed on a Shimadzu FT-IR IRTracer-100 spectrometer (Shimadzu Corporation, Tokyo, Japan) equipped with a KBr beamsplitter and a DLARGS detector, using a single-reflection diamond ATR crystal mounted on a ZnSe plate, with ATR performed in the range 4000–400  $\text{cm}^{-1}$ , and data collection being performed after 20 recordings at a resolution of 4  $\text{cm}^{-1}$ .

- TG/DTG analysis

Sample analysis was performed on a METTLER TOLEDO thermogravimetric analyzer, model TGA/DSC3+ (Mettler-Toledo LLC, Columbus, OH, USA), over a temperature range of 25–500 °C with a heating rate of 10 °C/min in air atmosphere (with a flow rate of 50 mL/min), in open aluminum crucibles.

- Bacterial Strains and Culture Conditions

The antibacterial activity of films was evaluated against *Staphylococcus aureus* ATCC 29213 (Gram-positive) and *Escherichia coli* ATCC 25922 (Gram-negative), both purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Frozen glycerol stocks were thawed on ice and streaked on Tryptic Soy Agar (TSA; 15 g/L agar, 15 g/L pancreatic digest of casein, 5 g/L sodium chloride, 5 g/L papain digest soy peptone; Merck). After 24 h incubation at 37 °C, a single colony from each strain was inoculated into Tryptic Soy Broth (TSB; 17 g/L pancreatic digest of casein, 3 g/L papain digest of soy peptone, 2.5 g/L dipotassium hydrogen phosphate, 2.5 g/L glucose, 5 g/L sodium chloride) and cultured overnight at 37 °C and 250 rpm in a thermostatic orbital shaker Heidolph Unimax 1010 (Heidolph Scientific Products, Schwabach, Germany).

- Antibacterial effect of amygdalin-doped films. TTC viability assay

Bacterial viability was assessed using the TTC (2,3,5-triphenyltetrazolium chloride) assay according to Moussa et al. [39], with minor modifications. A TTC stock solution (5 mg/mL) was prepared fresh, sterilized through a 0.22  $\mu\text{m}$  syringe filter, and stored at 4 °C for same-day use. TTC, a colorless tetrazolium salt, was reduced by intracellular dehydrogenases of metabolically active cells to red, insoluble formazan, which was subsequently solubilized in ethanol. Formazan production was quantified spectrophotometrically at 485 nm using a Biotek Synergy H1 microplate reader (Agilent, Santa Clara, CA, USA).

The antibacterial test was adapted from Galiano et al. [40] with minor modifications. Overnight cultures were diluted in TSB to an optical density of 0.1 at 600 nm ( $\text{OD}_{600}$ ). Aliquots of 5 mL were dispensed into six-well plates, and the polymeric films were immersed in the bacterial suspensions. Plates were incubated at 37 °C under agitation for 24 h. Control wells containing only the bacterial suspensions were processed in parallel.

After incubation, samples were removed, and 100  $\mu\text{L}$  of each bacterial suspension (samples and controls) was transferred to a sterile 96-well plate. Then, 50  $\mu\text{L}$  of TTC solution was added to each well, followed by incubation for 1 h in the dark under agitation. Plates were centrifuged at 4000 rpm for 5 min in a Hettich Universal 320 R centrifuge (Andreas Hettich GmbH, Tuttlingen, Germany), the supernatant discarded, and the formazan pellet resuspended and solubilized in 50% ethanol. A second centrifugation step (4000 rpm, 5 min) was performed, and the resulting supernatant was transferred to a fresh 96-well plate. Absorbance was recorded at 485 nm, and bacterial viability was calculated relative to the untreated control and expressed as percentage viability.

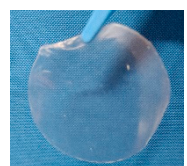
The statistical interpretation of results (ANOVA and Tukey HSD test) has been performed using Excel and an online calculator prepared by CC 2016 Navendu Vasavada ([https://astatsa.com/OneWay\\_Anova\\_with\\_TukeyHSD/](https://astatsa.com/OneWay_Anova_with_TukeyHSD/), accessed on 24 February 2026) [41].

### 3. Results and Discussion

#### 3.1. Synthesis of Amygdalin-Loaded Films

According to the synthesis protocol [28], five samples were obtained by the casting method, the polymer matrix of which is made up of three different biopolymers and a plasticizer (glycerol), one of which is the control sample, and is therefore without active substance ( $\kappa\text{Am}0$ ), and four in which different amounts of amygdalin were incorporated in increasing order ( $\kappa\text{Am}1$ – $\kappa\text{Am}4$ ). The amounts of incorporated active substance were 5 mg in the case of sample  $\kappa\text{Am}1$ , 10 mg for  $\kappa\text{Am}2$ , 15 mg for sample  $\kappa\text{Am}3$ , and 20 mg for sample  $\kappa\text{Am}4$ , respectively. Once cast, the samples were dried at a temperature of 45  $^{\circ}\text{C}$ , and their peeling from the Petri dish was possible after 48 h. It should be noted that once dried, the films were detached very easily from the Petri dish, maintaining their cast shape. They are completely transparent, without any visible trace of crystallized active substance, as can be seen in Table 1. Before the 48 h had elapsed, an attempt was made to peel off the control sample after 24 h of drying, but this was not possible, resulting in the tearing of a portion of the  $\kappa\text{Am}0$  film, as can be seen from its appearance. Therefore, the samples were left to dry for another 24 h, which allowed and facilitated peeling without affecting their integrity.

**Table 1.** Physical appearance of synthesized films.

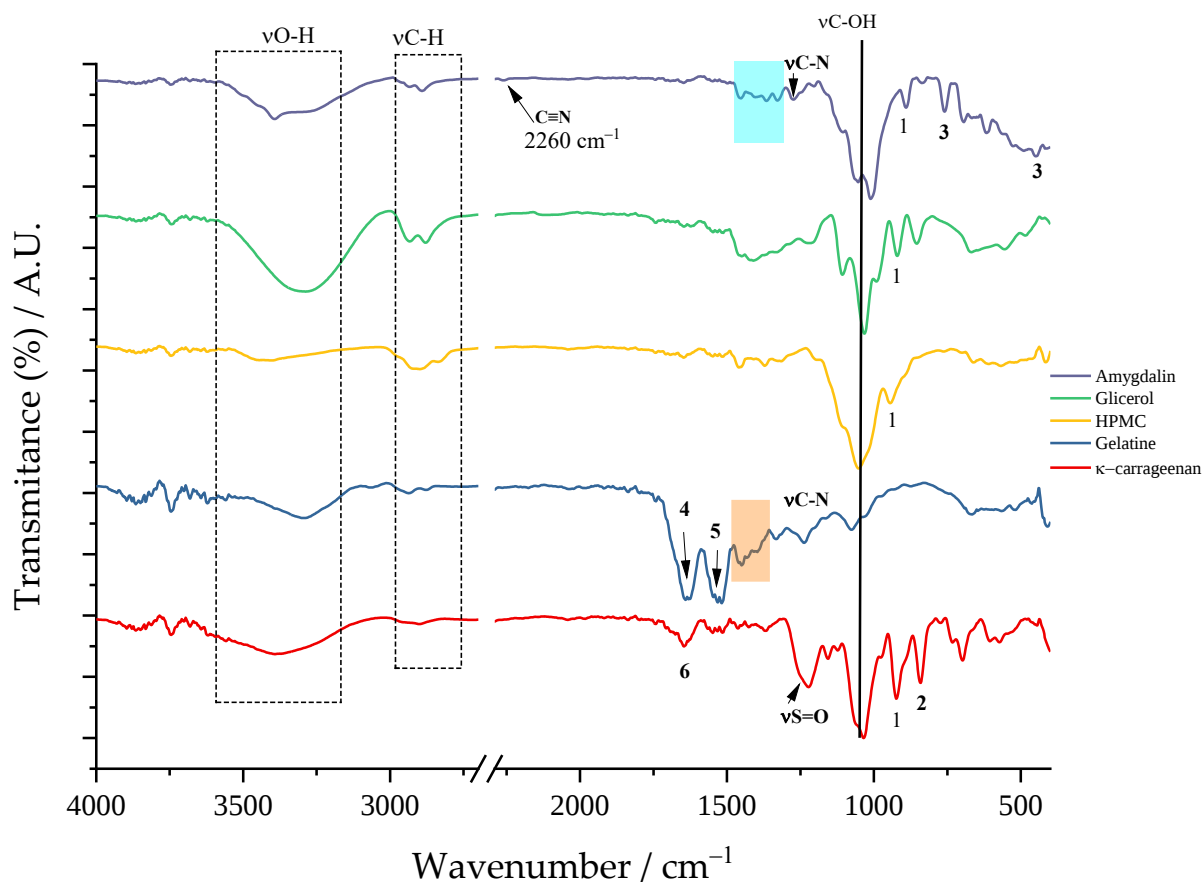
|                     | $\kappa\text{Am}0$                                                                  | $\kappa\text{Am}1$                                                                  | $\kappa\text{Am}2$                                                                   | $\kappa\text{Am}3$                                                                    | $\kappa\text{Am}4$                                                                    |
|---------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| Amygdalin (mg)      | 0                                                                                   | 5                                                                                   | 10                                                                                   | 15                                                                                    | 20                                                                                    |
| Physical appearance |  |  |  |  |  |

#### 3.2. FTIR Analysis

- Constituents of the film's biopolymer matrix

Starting from the marine biopolymer,  $\kappa$ -carrageenan (KC), according to Figure 1, it can be observed that it presents a very intense peak around 1000  $\text{cm}^{-1}$  which comes from the stretching vibration of the C–OH bond on the galactose units. The vibration of the glycosidic bonds ( $\nu\text{C–O–C}$ , designated no. 1) between these saccharide units leads to the appearance of a peak of average absorbance at 932  $\text{cm}^{-1}$ . The sulfated nature of this compound can be highlighted by two peaks, one at 1220  $\text{cm}^{-1}$  as a result of the S=O stretching vibration [42] and another at 842  $\text{cm}^{-1}$  due to  $\nu\text{C–OSO}_3\text{Na}$  (designated no. 2). Moreover, as can be seen in

the FTIR spectrum of this biopolymer, the peak from  $\approx 1652\text{ cm}^{-1}$  can be attributed to the bending vibration of the O-H group from the water bound by carrageenan [43] (designated with no. 6), thus confirming the hygroscopic property of  $\kappa$ -carrageenan mentioned in the literature [44]. The FTIR spectrum of hydroxypropyl methylcellulose (HPMC) can reconfirm the polysaccharide skeleton of this cellulose derivative both through the vibrations of the interatomic bonds of the  $\nu\text{C-OH}$  type at  $1056\text{ cm}^{-1}$  and the  $\nu\text{C-O-C}$  type at  $940\text{ cm}^{-1}$ . Peaks in the range of  $1300\text{--}1400\text{ cm}^{-1}$  can be attributed to deformation vibrations for both O-H and C-H from the saccharide units. Moreover, in the case of the HPMC sample, a side shoulder of the peak attributed to  $\nu\text{C-H}$  ( $2800\text{--}3000\text{ cm}^{-1}$ ) from the basic skeleton of the saccharide unit can be observed. This low-intensity peak, with some significance, indicates the stretching vibration for the terminal C-H, more precisely the methyl portion of the propyl fragment of the cellulose derivative, which could not be observed in the case of the other analyzed samples. As for the animal biopolymer, gelatine (GEL), the amino acid skeleton determines the appearance of the peak for the  $\nu\text{C=O}$  vibration at  $1635\text{ cm}^{-1}$  (Amide I band, designated no. 4), the deformation vibration of the N-H bond at  $1528\text{ cm}^{-1}$  (Amide II band, designated no. 5) [45,46] but also the stretching vibration for C-N at  $1239\text{ cm}^{-1}$  (Amide III band). It is also possible to observe (in the orange quadrant) in the area  $1300\text{--}1450\text{ cm}^{-1}$  the skeletal vibration of the  $\nu\text{C-C}$  bond due to the presence of the pyrrolidine ring; as a constituent fragment of this biopolymer, this region also corresponds to the hydrogen bending vibrations. In the FTIR spectrum of glycerol (GLY), it is possible to observe again the appearance of an intense peak around  $1028\text{ cm}^{-1}$  as a result of the stretching vibration of the C-OH bond, but also a broad band in the range  $1300\text{--}1400\text{ cm}^{-1}$  which includes the deformation of the interatomic O-H and C-H bonds.

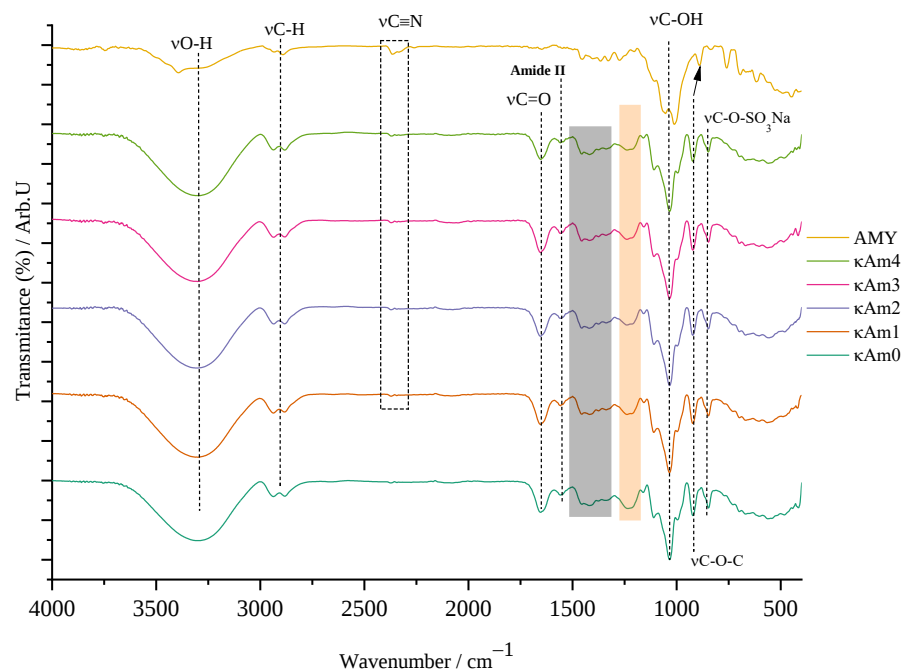


**Figure 1.** Graphical representation of FTIR spectra for the components of the biopolymer matrix of the films and the active substance (AMY).

In the FTIR spectrum of the AMY (Figure 1), the following peaks can be observed, which can be grouped as follows: peaks originating from the saccharide portion ( $\nu\text{C-OH}$  at  $1028\text{ cm}^{-1}$  and  $\nu\text{C-O-C}$  at  $900\text{ cm}^{-1}$ ); peaks originating from the aromatic portion (two peaks designated with no. 3, indicating the monosubstituted nature of the benzene ring, at  $760\text{ cm}^{-1}$  and at  $453\text{ cm}^{-1}$ ); peaks originating from the nitrile group. Although it was expected that the nitrile group in amygdalin would lead to the appearance of a very visible peak around  $2100\text{--}2250\text{ cm}^{-1}$ , in the present case, this peak is hardly visible. Instead, the stretching vibration of the C-N bond appears at  $1267\text{ cm}^{-1}$  even though this compound does not present any single C-N bond. A possible answer to this phenomenon could be the nature of the triple bond itself. Thus, it is possible that the vibration of the two constituent bonds (of the  $\pi$  type) is displaced and even “masked” by the skeletal vibration of the aromatic ring (blue quadrant) while the  $\sigma$  type bond is more easily detectable than the nitrile group itself, hence the unexpected appearance of the  $\nu\text{C-N}$  vibration in the case of amygdalin. Finally, it should be noted that all the analyzed samples present characteristic peaks for  $\nu\text{O-H}$  in the area  $3000\text{--}3500\text{ cm}^{-1}$  and for  $\nu\text{C-H}$  in the area  $2800\text{--}3000\text{ cm}^{-1}$ , respectively.

- Amygdalin-doped films

For a better interpretation of the FTIR spectra of the doped films, the spectrum of the control sample and of the active substance was also represented, according to Figure 2. Knowing that all synthesized films ( $\kappa\text{Am}0$ ,  $\kappa\text{Am}1$ ,  $\kappa\text{Am}2$ ,  $\kappa\text{Am}3$ , and  $\kappa\text{Am}4$ ) are based on three different types of biopolymers, characteristic peaks for each polymer can be found in their FTIR spectra. Thus, the presence of carrageenan can be highlighted through the peak that appears at  $846\text{ cm}^{-1}$  as a result of the vibration of the  $\nu\text{C-OSO}_3\text{Na}$  bond, this peak indicating the unaltered presence of the sulfonic group on the saccharide skeleton of this biopolymer in the analyzed samples. Also, this biopolymer, through the S=O bond but together with the C-N bond vibration (from GEL but also from AMY) contributes to the physical appearance of the band/peak in the range  $1180\text{--}1289\text{ cm}^{-1}$  found in the doped film samples. Upon closer inspection, it can be seen that this broad peak/band presents two slightly distinct peaks, the one at  $\approx 1212\text{ cm}^{-1}$  being given by  $\nu\text{S=O}$  and the one at  $1240\text{ cm}^{-1}$  in response to the presence of  $\nu\text{C-N}$ . Moreover, as the concentration of active substance in the films increases ( $\kappa\text{Am}1 < \kappa\text{Am}2 < \kappa\text{Am}3 < \kappa\text{Am}4$ ), a slight tendency for broadening and flattening of this band can be observed, but only in the portion given by the C-N vibration. Thus, the increase in the AMY concentration determines an “enrichment” of the system with  $\sigma$  bonds of C-N nature. Therefore, this portion of the curve (in the orange quadrant) tends towards the “shape/appearance” of the  $\nu\text{C-N}$  peak in the FTIR spectrum of the pure active substance (Figure 1). In the case of GEL, as a constituent of the biopolymer matrix of the films, it can be highlighted through the vibration of the pyrrolidine ring in the range of  $1300\text{--}1400\text{ cm}^{-1}$  (gray quadrant) but also through the stretching vibration of the carbonyl of the peptide bond (amide band I) at  $1652\text{ cm}^{-1}$  as well as the  $\delta\text{N-H}$  vibration (amide band II) at  $1548\text{ cm}^{-1}$ , these two vibrations being found in all the analyzed films. As for HPMC and GLY, it can be said that these components enrich the system with C-OH, O-H, and C-H type bonds, thus determining an intensification of the corresponding peaks in the analyzed samples, more than would be the case for each component separately (in Figure 1). The presence of the active substance in the synthesized films can be brought to the fore through the stretching vibration peak of the nitrile group at  $2337\text{ cm}^{-1}$  as well as through the peak denoting the monosubstituted nature of this compound, at  $424\text{ cm}^{-1}$ , a clear exception being the control sample ( $\kappa\text{Am}0$ ). Also, the saccharide portion of amygdalin contributes to the intensity of the peaks for  $\nu\text{C-OH}$ ,  $\nu\text{O-H}$ , and  $\nu\text{C-H}$ , respectively.



**Figure 2.** FTIR spectra of the AMY-doped films ( $\kappa$ Am1,  $\kappa$ Am2,  $\kappa$ Am3, and  $\kappa$ Am4), the active substance (AMY), and the control sample ( $\kappa$ Am0).

### 3.3. TG/DTG Analysis

- Constituents of the film's biopolymer matrix

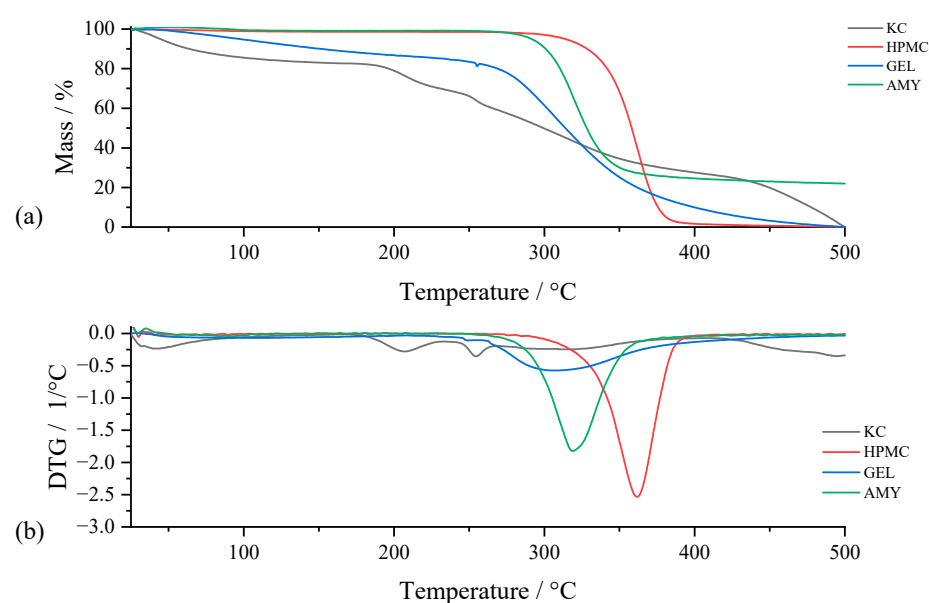
In order to understand the thermal behavior of the films doped with AMY, the first step was to analyze the constituents of the polymer matrix. Thus, for the components that enter into their composition, the three biopolymers, but also the active substance, the results obtained from the thermogravimetric analysis are represented in Figure 3. The tabular representation, which includes mass loss,  $T_{\text{onset}}$ ,  $T_{\text{endset}}$ , and  $T_{\text{max}}$  for each analyzed sample, is given at the end of this section, in Table 2. The most important constituent of the biopolymer matrix, KC, presents four thermal stages, following which the total mass loss is 38.03%. As can be observed on the DTG curve of this biopolymer (Figure 3b), the first thermal process associated with moisture loss is located in a temperature range lower than 50 °C. This is followed by the elimination of water “bound” to the biopolymer, which, as mentioned earlier, is also “visible” in its FTIR spectrum. The thermal process associated with the elimination of “bound” water is located in the temperature range of 160–210 °C. The decomposition of KC, which starts by the elimination of the sulfonic group from the saccharide skeleton [47], is located in the third thermal process, with a mass loss of  $\approx 7\%$  and for which the  $T_{\text{max}}$  is located at  $\approx 250$  °C. The proper decomposition of this biopolymer takes place in the fourth process, and, as expected, presents the greatest mass loss, of 15.54%.

For the HPMC component, the DTG curve highlights a single thermal decomposition process, located in the temperature range of 274.37–431.35 °C, with a maximum of the process located at 335.55 °C and a total mass loss of 89.10%. This single process indicates the lack of water as moisture in this component, and as suggested by the literature [48,49], this single process may indicate the purity of HPMC; otherwise, impurities may contribute to the occurrence of secondary thermal processes. Moreover, following our published works so far [28,50], it was observed that cellulose derivatives tend to present a single decomposition process (such as the case of carboxymethyl cellulose and hydroxyethyl cellulose). For the GEL sample, three thermal processes can be distinguished, resulting in a total mass loss of 55.41%. The thermal process within the temperature range of

40.14–98.79 °C is due to moisture loss, and the two thermal processes, to which the highest mass loss of 55.28% is associated, are the result of the actual decomposition of the gelatin skeleton [51,52], which includes different types of oxidation reactions of the constituent amino acids. The thermal analysis of the active substance, AMY, highlights two thermal processes, resulting in a mass loss of 75.18%. The actual decomposition of the active substance occurs within the most pronounced thermal process, in the temperature range of 264.35–390.21 °C, while moisture loss occurs within the first thermal process of the DTG curve (Figure 3).

- Amygdalin-doped films

The results of the thermal analysis indicate the presence of four distinct thermal processes, both for the active substance-doped films and for the control one, as can be seen in Figure 4. Thus, for the  $\kappa$ Am0 sample, the first thermal process corresponds to the loss of moisture, in the temperature range of 40.33–85.54 °C, and a mass loss of 5.06%. This is followed by the process attributed to the elimination of water captured in the biopolymer matrix, where the mass loss is 53.10% for a maximum of the process located at 147.52 °C. Moreover, as indicated in the literature, the volatilization of the plasticizer, glycerol, also takes place in this process [52]. By examining the mass loss associated with this thermal process (Table 2) for each sample, we can see that mass loss gradually decreases as the amount of incorporated amygdalin increases. This downward trend suggests that higher amygdalin concentrations may help the polymer matrix maintain its structure. The reduced mass loss likely reflects a lower release of polymer-bound water, indicating better hydration and a more stable network. In this context, amygdalin may enhance intermolecular interactions, such as hydrogen bonding, contributing to improved thermal stability and reduced susceptibility to dehydration. According to the positioning of the third thermal process, its associated maximum can be attributed to the initiation of the decomposition of  $\kappa$ -carrageenan by the elimination of the sulfonic group [53]. This process presents a mass loss of 8.64% with the temperature limits of  $T_{\text{onset}}$  at 237.50 °C and  $T_{\text{endset}}$  at 294.54 °C. The last thermal process, although of low intensity, is in a wide temperature range (301.04–371.82 °C) and can be attributed to the actual decomposition of the control sample.



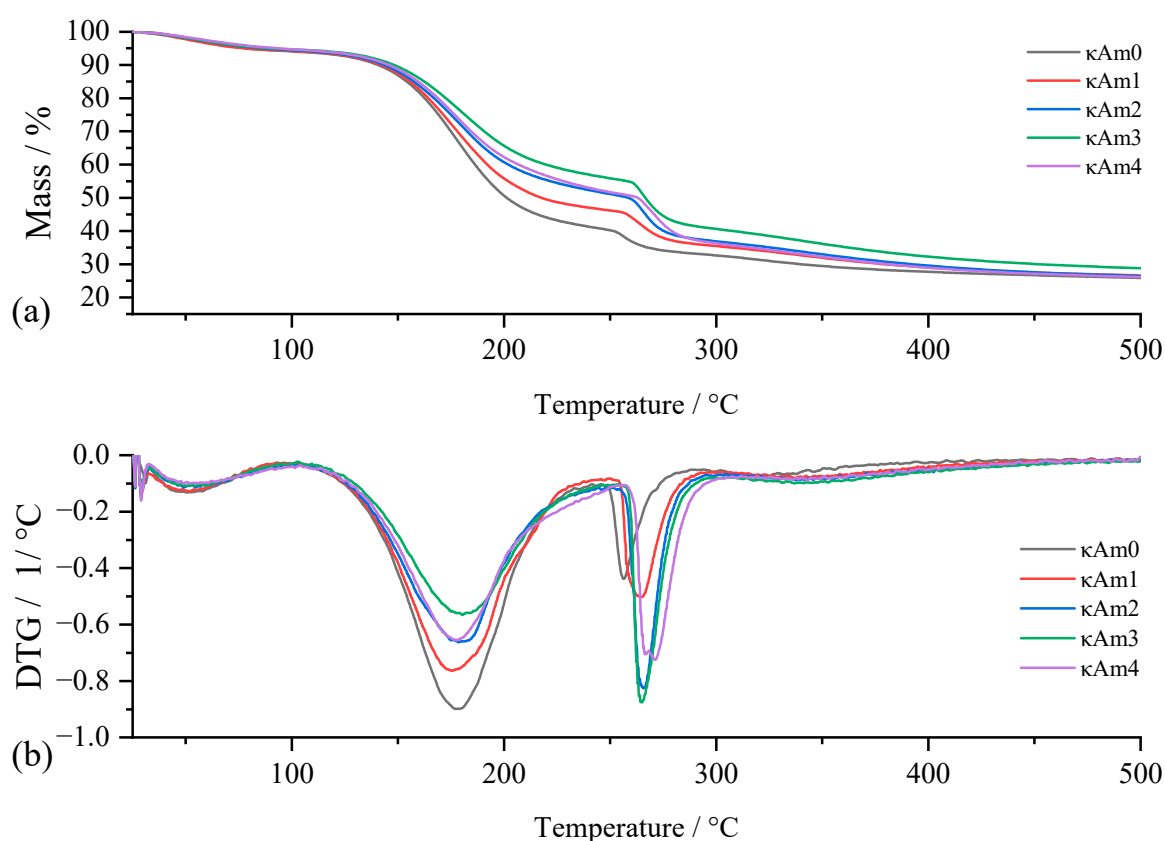
**Figure 3.** Graphical representation of thermal analysis data for the constituents of the biopolymer matrix of the films (corresponding TG (a) and DTG (b) curves).

**Table 2.** Thermoanalytical data for the basic components of the samples, the control ( $\kappa$ Am0), and the AMY-doped films ( $\kappa$ Am1– $\kappa$ Am4).

| Sample       | Process No. | Mass Loss (%) | DTG Temperature (°C) |                  |                     |
|--------------|-------------|---------------|----------------------|------------------|---------------------|
|              |             |               | T <sub>onset</sub>   | T <sub>max</sub> | T <sub>endset</sub> |
|              | 1           | −9.46         | 36.65                | 64.39            | 96.79               |
|              | 2           | −6.93         | 167.45               | 193.25           | 216.96              |
|              | 3           | −6.10         | 228.45               | 249.51           | 260.08              |
|              | 4           | −15.54        | 270.30               | 289.55           | 339.22              |
| GEL          | 1           | −0.13         | 40.14                | 61.20            | 98.79               |
|              | 2           | −0.69         | 255.66               | 256.95           | 258.35              |
|              | 3           | −54.59        | 259.41               | 359.08           | 439.67              |
| HPMC         | 1           | −89.10        | 274.37               | 335.55           | 431.35              |
| AMY          | 1           | −1.47         | 42.09                | 55.72            | 129.23              |
|              | 2           | −73.71        | 264.35               | 347.35           | 390.21              |
|              | 1           | −5.06         | 40.33                | 68.64            | 85.54               |
|              | 2           | −53.10        | 101.19               | 147.52           | 224.26              |
|              | 3           | −8.64         | 237.50               | 268.77           | 294.54              |
|              | 4           | −4.07         | 301.04               | 343.04           | 371.82              |
| $\kappa$ Am1 | 1           | −5.05         | 39.83                | 70.43            | 92.04               |
|              | 2           | −46.82        | 99.01                | 147.38           | 227.40              |
|              | 3           | −11.91        | 241.39               | 279.14           | 307.93              |
|              | 4           | −7.10         | 303.25               | 379.06           | 423.49              |
|              | 1           | −5.25         | 41.27                | 77.99            | 106.30              |
|              | 2           | −42.93        | 104.04               | 146.06           | 229.33              |
|              | 3           | −14.93        | 248.54               | 278.62           | 307.43              |
|              | 4           | −6.02         | 311.32               | 365.57           | 391.02              |
| $\kappa$ Am3 | 1           | −4.99         | 39.29                | 75.51            | 101.82              |
|              | 2           | −38.65        | 106.86               | 148.80           | 231.70              |
|              | 3           | −15.34        | 250.40               | 279.69           | 301.98              |
|              | 4           | −8.37         | 311.58               | 376.84           | 417.62              |
|              | 1           | −5.17         | 40.92                | 78.97            | 107.80              |
|              | 2           | −43.10        | 111.58               | 148.37           | 236.30              |
|              | 3           | −15.75        | 256.55               | 284.63           | 315.11              |
|              | 4           | −7.04         | 319.53               | 379.61           | 428.09              |

In the case of films containing active substance ( $\kappa$ Am1– $\kappa$ Am4), the DTG curve also highlights four thermal processes which, as can be seen from Figure 4b, differ from the control sample by their intensity. Thus, for the sample containing the smallest amount of active substance ( $\kappa$ Am1), the elimination of moisture is accompanied by a mass loss of 5.06%, at a T<sub>max</sub> of the process of 70.43 °C while the water “trapped” by the matrix is eliminated in the temperature range of 99.01–227.40 °C, with a mass loss of 46.82 °C. Once this process is completed, the disintegration of the basic biopolymer begins, with a mass loss of 11.91% at a T<sub>max</sub> of the process located at 279.14 °C. The continuous decomposition of the polymer matrix and the active substance is included in the fourth thermal process, at temperatures >300 °C with a mass loss of 7.10%. For the  $\kappa$ Am2 sample, the loss of water in the form of moisture and that water trapped in the polymer matrix is accompanied by a mass loss of 5.25% at a T<sub>max</sub> of 77.99 °C and 42.93% at a T<sub>max</sub> of 146.06 °C, respectively. The actual decomposition of the film induces a mass loss of 20.95%, in the range of 248.54–391.02 °C, encompassing the last two thermal processes. In the case of the  $\kappa$ Am3 sample, in the temperature range of 39.29–101.82 °C, moisture loss occurs through a mass loss of 4.99%, while the elimination of “bounded” water by the biopolymers is accompanied by a mass loss of 38.65% for a T<sub>max</sub> of the process located at 148.80 °C. The beginning of the decomposition of carrageenan is given by the process corresponding to the breaking of the sulfonic group with a mass loss of 15.34% in the temperature range of 250.40–301.98 °C. This

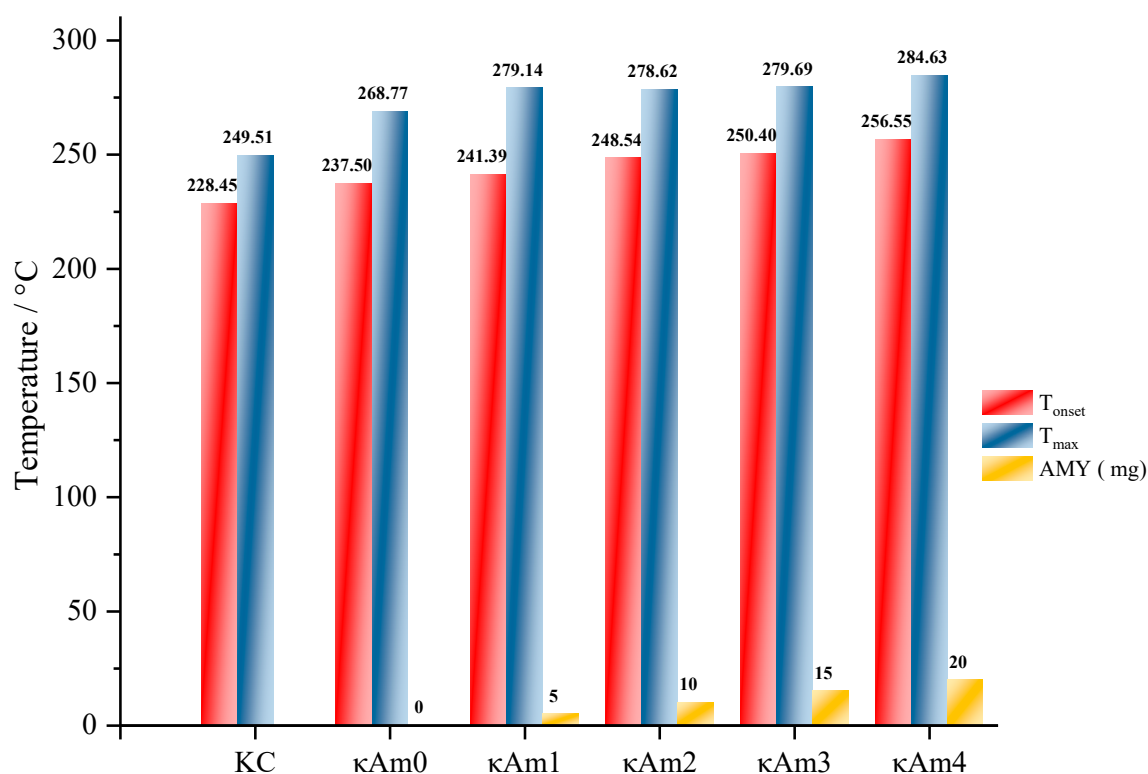
decomposition continues in the fourth thermal process along with the other components of the analyzed sample for which the mass loss is 8.37%. Finally, the four thermal processes can also be found in the case of the sample that presents the highest amount of incorporated active substance, the  $\kappa$ Am4 sample. Thus, the first thermal process in which the mass loss is 5.17% can be attributed to the loss of water in the form of humidity. Within the second thermal process, there is an elimination of water bound to the polymer matrix where it is accompanied by a mass loss of 43.10% in the temperature range of 111.58–236.30 °C. Once it is completed, the matrix disintegration begins; first the elimination of the sulfonic group occurs within the third process, followed by the actual decomposition of the analyzed film. Thus, the mass loss for the third thermal process is 15.75% and 7.04% for the last thermal process observable on the DTG curve of the film in question.



**Figure 4.** Graphical representation of thermal analysis data for amygdalin-doped films (corresponding TG (a) and DTG (b) curves).

By analyzing the thermoanalytical data, presented in Table 2, we can observe a slight increase in the  $T_{\text{onset}}$  value of the thermal process associated with the elimination of the sulfonic group from carrageenan compared to the value of the same process in the case of the pure carrageenan sample (KC). Knowing that in the synthesized film samples, only the amount of incorporated active substance has been varied, we can say that the increase in the  $T_{\text{onset}}$  value is determined by amygdalin itself. Moreover, the amount of incorporated amygdalin has a similar effect on the  $T_{\text{max}}$  values of the same thermal process. Thus, it can be deduced that the increase in the amygdalin concentration increases the thermal stability of the polymer matrix, having a direct impact on the thermal stability of the polymer matrix of the base polymer,  $\kappa$ -carrageenan. In addition, if we compare the values of the same process, for the control sample ( $\kappa$ Am0) and for pure carrageenan, we can observe a difference between the  $T_{\text{onset}}$  and  $T_{\text{max}}$  values. Even though the control sample does not contain active substance, a difference of even 19 °C can be seen, which indicates that

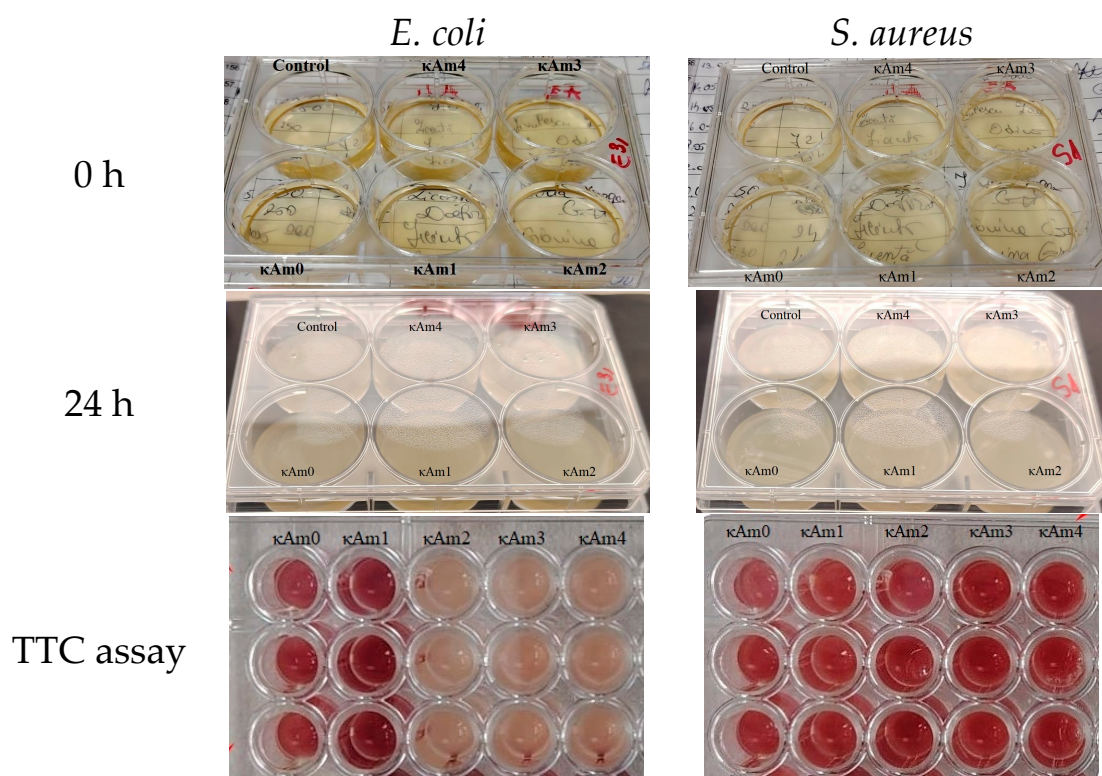
GEL and HPMC in the control film also have an impact on the thermal stability of the synthesized films. Having said that, it can be concluded that the greatest impact on the thermal stability of the patches is given by AMY because its presence leads to a 10 °C increase in the value of the mentioned parameters, including at its the lowest concentration (sample  $\kappa$ Am1), compared to the control sample. Subsequently, a slight plateau of the  $T_{\text{onset}}$  and  $T_{\text{max}}$  values can be observed, which may suggest a possible “reaching” of the upper limit beyond which the concentration of the active substance can no longer influence the thermal stability of the films, the parameter values no longer having a linear increase. For a better and more efficient “visualization” of the communicated information, the  $T_{\text{max}}$  and  $T_{\text{onset}}$  values of the synthesized films are compared to those of pure carrageenan and represented graphically in Figure 5.



**Figure 5.** Graphical representation of the  $T_{\text{onset}}$  and  $T_{\text{max}}$  values of the process corresponding to the initiation of carrageenan decomposition, depending on the synthesized film and the concentration of incorporated active substance.

### 3.4. In Vitro Evaluation

The antibacterial activity testing of the synthesized patches was carried out in two series, including a control sample. The synthesized patches were incubated at 37 °C for 24 h, in direct contact with the culture medium, both for the *E. coli* and *S. aureus* cultures, after which, according to the test methodology, the bactericidal efficiency and viability, respectively, were determined spectrophotometrically. Figure 6 shows the appearance of the inoculated samples at  $t_0$  of incubation—the moment of their contact with the bacterial cultures—and at  $t_{24}$ —after 24 h of contact with the bacterial cultures—as well as their chromatic response to the TTC assay.

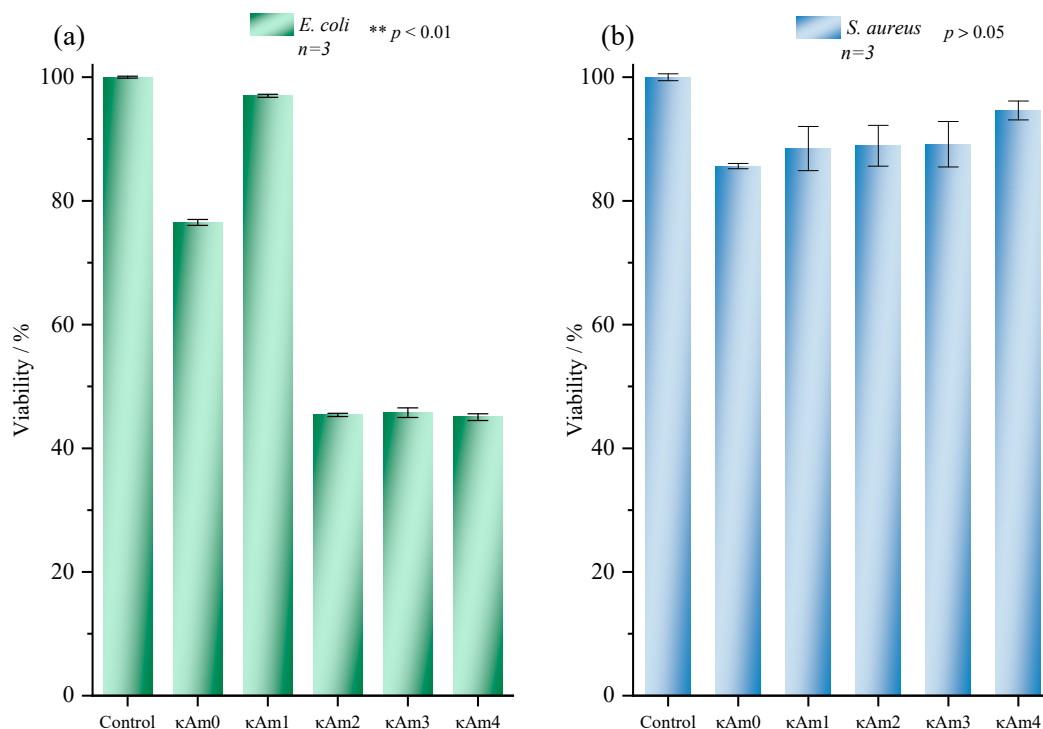


**Figure 6.** Physical appearance of the samples at the time of contact with the culture medium ( $t = 0$  h), after 24 h of contact with the culture medium ( $t = 24$  h) and the coloration of the complex in the TTC assay.

Regarding the viability test, for both *E. coli* and *S. aureus* cultures, results are graphically represented in Figure 7. Thus, as can be seen, in the case of both bacterial cultures, the control film ( $\kappa\text{Am}0$ ) shows a slight bactericidal activity, the viability being  $\approx 80\%$ , and efficiency being  $\approx 20\%$ , respectively. Knowing that the control sample does not contain an active substance, there is a possibility that one of the components of the polymer matrix of the film may show slight antibacterial activity. As the literature data indicate [54,55], carrageenan exhibits remarkable antibacterial properties, including activity against *E. coli* and *S. aureus* [56,57]. Therefore, the bactericidal efficiency of the control sample against the studied pathogens may be due to this marine biopolymer.

For the AMY-doped films tested on the *E. coli* strain, as can be seen from the chromatic appearance of the samples in the TTC assay (Figure 6), they exhibit significant bactericidal activity against this pathogen. Comparing the results of the viability test for the amygdalin-doped patches, it was expected that the lowest concentration of incorporated active substance (sample  $\kappa\text{Am}1$ ) would show higher efficiency than the control sample; however, the  $\kappa\text{Am}1$  sample shows a reduction in efficiency, with a cell viability of 97.5%, corresponding to 2.5% inhibition. A possible explanation for this phenomenon could be the concentration of the incorporated active substance. Thus, there is the possibility that the amount of AMY in the sample  $\kappa\text{Am}1$  is insufficient to inhibit bacterial growth; rather, it becomes a nutrient for bacteria, knowing that *E. coli* possesses glucosidases [58] that may be able to cleave the glycosidic bond from the saccharide units present in AMY. In this case, AMY as a nutrient increases the multiplication of bacteria, the large number of which exceeds the capacity of carrageenan to exert its bactericidal property, hence the low value of the viability test efficiency of this film. Another rather interesting observation is the behavior of the  $\kappa\text{Am}2$ ,  $\kappa\text{Am}3$ , and  $\kappa\text{Am}4$  samples. These samples far exceed the bactericidal efficiency of the control sample and the  $\kappa\text{Am}1$  sample, as can be seen from Figure 7a.

Therefore, it can be concluded that increasing the amount of active substance enhances the bactericidal effect of the patches in question, achieving an efficiency of  $\approx 54\%$  against the *E. coli* strain. It can also be observed that, regardless of the increase in the concentration of active substance (AMY), the bactericidal efficiency peaks around the value of 54% (54.29% for  $\kappa\text{Am}2$ , 53.94% for  $\kappa\text{Am}3$ , and 54.66% for  $\kappa\text{Am}4$ ). The increase in bactericidal activity is therefore determined by the AMY concentration in the respective samples and not by carrageenan, greatly exceeding the efficiency of the control sample ( $\kappa\text{Am}0$ ). Reaching the “limit threshold” of efficiency for the  $\kappa\text{Am}2$ ,  $\kappa\text{Am}3$ , and  $\kappa\text{Am}4$  samples may suggest that the active substance cannot eliminate 100% of *E. coli* bacteria but rather can “maintain” and control within reasonable limits their number and ability to multiply.



**Figure 7.** Schematic representation of the viability test results of film samples tested on *E. coli* (a) and *S. aureus* (b) cultures compared to the pathogen control sample (Control—culture medium + bacterial inoculum), \*\* meaning very significant.

In the case of AMY-doped films tested on *S. aureus*, there is an opposite behavior to that observed from the test on the *E. coli* strain. According to Figure 7b, the increase in the concentration of AMY in the analyzed samples leads to a decrease in the bactericidal efficiency against *S. aureus*, following the order  $\kappa\text{Am}0 > \kappa\text{Am}1 > \kappa\text{Am}2 > \kappa\text{Am}3 > \kappa\text{Am}4$ , with the  $\kappa\text{Am}4$  sample reaching an efficiency of only 5.36%. In our previously published study [28], it was observed that the nature of the biopolymers present in the polymeric matrix of the patches (combined with carrageenan) can influence the bactericidal efficiency response. The combination of carrageenan with carboxymethyl cellulose resulted in a low bactericidal efficiency against the same pathogens, as was also observed in the present study. Therefore, as was the case in the previous study, it is possible that the cellulose derivative, in this case hydroxypropyl methylcellulose, is susceptible to attack by *S. aureus*, which, through its phospho- $\beta$ -glucosidases [59], “attacks” both the active substance and the biopolymer matrix of the films. Once subjected to attack, they practically become a nutrient for *S. aureus*, promoting the multiplication of this pathogen, thus preventing the active substance from exerting its bactericidal activity. The potential use of the HPMC from the film matrix as a nutrient by *S. aureus* represents a limitation of the current system and could

partially explain the observed reduction in bactericidal efficacy. A possible improvement in the antibacterial efficacy of amygdalin-doped films against *S. aureus* culture may be achieved by modifying the polymeric matrix, including crosslinking the HPMC to prevent its enzymatic degradation or modifying the combination ratio between the constituents of the biopolymer matrix.

Following the unifactorial ANOVA analysis, for the patches tested on the *E. coli* culture, the data highlight a significant effect of the concentration of the active substance on bacterial viability, with a  $p$  of  $4.12 \times 10^{-15}$ . Furthermore, the Tukey post hoc test indicates significant differences between the control sample ( $\kappa$ Am0) and the samples containing entrapped amygdalin but also significant differences between the  $\kappa$ Am1 sample and the other samples with amygdalin (As can be seen in Table 3). The results of the Tukey test suggest/highlight a dose-dependent antibacterial effect, thus reconfirming the results of the viability test (Figure 7). A  $p$  value  $< 0.01$  indicates significant differences between the tested pairs, while viability reaches a “plateau” in the case of samples  $\kappa$ Am2,  $\kappa$ Am3, and  $\kappa$ Am4. The latter presents an insignificant difference between them, with the  $p$  value being much higher than 0.05. This “plateau” of bactericidal activity indicates a possible reaching of the “maximum” concentration of amygdalin capable of acting bactericidally on the tested pathogen. In conclusion, the results of the Tukey test could indicate that an increase in the concentration of amygdalin, above those tested, will not increase the bactericidal effect of this substance on *E. coli* cultures.

**Table 3.** Unifactorial ANOVA (a) and Tukey HSD (b) results for patches tested on *E. coli* culture.

| (a) ANOVA                     |                       |                      |                        |
|-------------------------------|-----------------------|----------------------|------------------------|
| Source of Variation           | df                    | F                    | $p$ -Value             |
| Between Groups                | 4                     | 2691.644             | $4.12 \times 10^{-15}$ |
| Within Groups                 | 10                    |                      |                        |
| Total                         | 14                    |                      |                        |
| (b) Tukey HSD Test [41]       |                       |                      |                        |
| Treatments Pair               | Tukey HSD Q Statistic | Tukey HSD $p$ -Value | Tukey HSD Inference    |
| $\kappa$ Am0 vs. $\kappa$ Am1 | 44.7077               | 0.0010053            | ** $p < 0.01$          |
| $\kappa$ Am0 vs. $\kappa$ Am2 | 67.8498               | 0.0010053            | ** $p < 0.01$          |
| $\kappa$ Am0 vs. $\kappa$ Am3 | 67.0887               | 0.0010053            | ** $p < 0.01$          |
| $\kappa$ Am0 vs. $\kappa$ Am4 | 68.6380               | 0.0010053            | ** $p < 0.01$          |
| $\kappa$ Am1 vs. $\kappa$ Am2 | 112.5574              | 0.0010053            | ** $p < 0.01$          |
| $\kappa$ Am1 vs. $\kappa$ Am3 | 111.7964              | 0.0010053            | ** $p < 0.01$          |
| $\kappa$ Am1 vs. $\kappa$ Am4 | 113.3457              | 0.0010053            | ** $p < 0.01$          |
| $\kappa$ Am2 vs. $\kappa$ Am3 | 0.7611                | 0.8999947            | insignificant          |
| $\kappa$ Am2 vs. $\kappa$ Am4 | 0.7883                | 0.8999947            | insignificant          |
| $\kappa$ Am3 vs. $\kappa$ Am4 | 1.5493                | 0.7862805            | insignificant          |

\*\* meaning very significant.

In contrast, for the samples tested on *S. aureus* culture, the results of the ANOVA analysis do not reveal significant statistical differences between the samples with different concentrations of amygdalin given that  $p$  is 0.33 (according to Table 4). Thus, it can be said that the evolution of bacterial viability observed in the case of the tested film samples may suggest that either amygdalin does not induce a bactericidal effect in the range of concentrations tested or as mentioned earlier, it may become susceptible to attack by the tested pathogen along with HPMC.

**Table 4.** Unifactorial ANOVA results for patches tested on *S. aureus* culture.

| Source of Variation | ANOVA |          |          |
|---------------------|-------|----------|----------|
|                     | df    | F        | p-Value  |
| Between Groups      | 4     | 1.303671 | 0.332849 |
| Within Groups       | 10    |          |          |
| Total               | 14    |          |          |

While the experimental evidence regarding the antibacterial mechanisms of amygdalin is still very low, there are some studies that demonstrate the inhibitory effect of amygdalin on bacterial species. For example, the study of Ajeel et al. [60] showed that the antibacterial activity of amygdalin has been reported to be concentration-dependent, with higher concentrations producing larger inhibition zones against various Gram-positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative (*Pseudomonas aeruginosa* and *Serratia marcescens*) bacterial strains after 24 h of incubation. In contrast, ethanol 35% used as a control (solvent used for solubilizing amygdalin) did not exhibit inhibitory effects. Following the well diffusion method, the results showed that all microorganisms were resistant to 2% amygdalin, and the inhibition effect was directly proportional to amygdalin concentration. Therefore, all other concentrations tested (4%, 6%, 8% and 10%) inhibited bacterial growth, with the inhibition zone diameter increasing as the amygdalin concentration increased [60].

The proposed mechanism underlying this activity is associated with the enzymatic hydrolysis of amygdalin by  $\beta$ -glucosidase, leading to the formation of D-glucose, benzaldehyde, and hydrogen cyanide [60–62].  $\beta$ -glucosidase activity has been identified in certain bacterial species, suggesting that amygdalin may be metabolized by microorganisms [63]. One hypothesis is that bacteria initially utilize glucose moiety as a nutrient source; however, the concomitant release of toxic degradation products may ultimately inhibit bacterial growth. Hydrogen cyanide is considered a key contributor to the antibacterial effect. Its toxicity is primarily attributed to the cyanide ion, which interferes with cellular respiration by inhibiting cytochrome c oxidase within the electron transport chain located in the bacterial membrane. This inhibition disrupts ATP production and leads to impaired cellular function. Unlike some fungi that possess detoxification mechanisms such as formamide hydrolyase, most bacteria lack efficient pathways to metabolize or neutralize cyanide, rendering them susceptible to its toxic effects [60,62,63]. In addition to hydrogen cyanide, benzaldehyde—another hydrolysis product—has also been suggested to contribute to antibacterial activity. Benzaldehyde may exert inhibitory effects through interactions with cellular components, including phenolic compounds, leading to the formation of toxic derivatives that further compromise bacterial viability [64].

A study by Abtahi et al. [62] confirmed the antibacterial effect of aqueous and alcoholic amygdalin extracts. Minimum inhibitory concentration (MIC) values of bitter apricot seed extracts against five bacterial strains demonstrated variable susceptibility among the tested microorganisms. Bacterial growth after 72 h of incubation in the presence of extract concentrations ranging from 2 to 512  $\mu\text{g/mL}$  was compared with untreated controls. For the methanolic extract, MIC values were reported as 128  $\mu\text{g/mL}$  for *Escherichia coli*, 32  $\mu\text{g/mL}$  for *Staphylococcus aureus*, 64  $\mu\text{g/mL}$  for *Salmonella typhi*, 32  $\mu\text{g/mL}$  for *Salmonella paratyphi* A, and 128  $\mu\text{g/mL}$  for *Salmonella paratyphi* B. In contrast, the aqueous extract exhibited lower MIC values, indicating enhanced antimicrobial potency, with MICs of 64  $\mu\text{g/mL}$  for *E. coli*, 16  $\mu\text{g/mL}$  for *S. aureus*, 32  $\mu\text{g/mL}$  for *S. typhi*, 32  $\mu\text{g/mL}$  for *S. paratyphi* A, and 64  $\mu\text{g/mL}$  for *S. paratyphi* B. The aqueous extract exhibited greater antibacterial activity compared to the methanolic extract, as reflected by lower MIC values across most tested strains. Although

the extract demonstrated measurable antimicrobial activity, its potency was generally lower than that of gentamicin, particularly against *Staphylococcus aureus* and *Salmonella typhi*. However, in certain cases such as *Escherichia coli* and *Salmonella paratyphi* B, the aqueous extract showed comparable or even lower MIC values than gentamicin, indicating noteworthy antibacterial potential. When compared with oxacillin, the extract exhibited superior activity against Gram-negative strains, which is consistent with the limited efficacy of oxacillin against bacteria possessing an outer membrane barrier. These findings suggest that bioactive compounds present in bitter apricot seeds may contribute to a relatively broad antimicrobial spectrum. The antibacterial activity of the extract is likely associated with the presence of secondary metabolites such as phenolic compounds, terpenoids, and alkaloids, which are known to interfere with microbial membrane integrity and metabolic processes [62]. Overall, the available literature suggests that the antibacterial activity of amygdalin is likely mediated by its enzymatic decomposition into bioactive compounds, particularly hydrogen cyanide and benzaldehyde, which interfere with essential bacterial metabolic processes.

Also, while testing the inhibitory effect of amygdalin on methicillin-resistant *Staphylococcus aureus* (MRSA), Wang et al. [65] showed that the MIC value of amygdalin to MRSA was 64 µg/mL. To evaluate the impact of amygdalin on MRSA resistance and virulence determinants, the expression of selected genes was analyzed by qRT-PCR following treatment. The results indicated that amygdalin reduced the transcription levels of biofilm-associated genes, including *sarA*, *fnbB*, and *icaA*. Additionally, the expression of virulence-related genes *hla* and *spA* was decreased after treatment, although statistical significance compared to the untreated MRSA group was observed only for *spA*. Amygdalin also significantly suppressed the expression of the  $\beta$ -lactamase-encoding gene *blaZ*. Although a reduction in *mecA* expression was observed, the difference was not statistically significant compared with the MRSA control. The effect of amygdalin on biofilm formation and disruption was further investigated using crystal violet staining. Treatment with amygdalin at 1/2 MIC significantly inhibited biofilm growth relative to the MRSA group, and this inhibitory effect increased with rising concentrations. Even at 1/8 MIC, a significant reduction in biofilm biomass was detected compared to the untreated control, suggesting a concentration-dependent capacity of amygdalin to interfere with biofilm development and promote biofilm clearance. Collectively, these findings suggest that amygdalin may exert antibacterial effects not only by inhibiting bacterial growth but also by modulating the expression of resistance- and biofilm-associated genes and impairing biofilm formation. Cell adhesion assays demonstrated that amygdalin reduced the ability of MRSA to adhere to A549 epithelial cells in a concentration-dependent manner. Compared with the untreated model group, treatment with low, medium, and high doses of amygdalin significantly decreased bacterial adhesion, with a more pronounced effect observed at higher concentrations. Levofloxacin also significantly reduced MRSA adhesion relative to the control group. However, the combination of high-dose amygdalin with levofloxacin did not result in a statistically significant additional reduction in adhesion compared with high-dose amygdalin alone, indicating no evident synergistic effect in this assay. Invasion assays further showed that amygdalin attenuated the capacity of MRSA to invade A549 cells. Although levofloxacin reduced invasion efficiency compared with the model group, the difference was not statistically significant. Among the amygdalin-treated groups, only the high-dose treatment produced a significant reduction in bacterial invasion relative to the control. Notably, the combined administration of amygdalin and levofloxacin exhibited a stronger inhibitory effect on MRSA invasion than either treatment alone, suggesting a potential enhancement of anti-invasive activity under combination conditions [65].

It should be noted that classical MIC and MBC determinations are typically performed for free antimicrobial agents in solution. In the present study, amygdalin was incorporated within a ternary biopolymer film system, where antibacterial performance is influenced not only by the intrinsic activity of the compound but also by matrix composition, diffusion processes, and release kinetics. Consequently, standard MIC/MBC values for free amygdalin would not fully represent the behavior of the developed composite films. Further quantitative evaluation of release-dependent antimicrobial parameters represents an important direction for future investigations.

#### 4. Conclusions

The need to develop new biopolymer drug carriers in the field of wound dressings led this research to the development of novel ternary biopolymer films as carriers for amygdalin. Due to their accessibility, biocompatibility, and versatility,  $\kappa$ -carrageenan, hydroxypropyl methylcellulose, and gelatin were selected as the polymer matrix components. The integration of the cellulose derivative hydroxypropyl methylcellulose into the polymer matrix of the films led to the formation of thin, elastic, and transparent structures that maintain their molded shape even when doped with amygdalin. In contrast, based on observations from previous research, it was observed that the combination ratio as well as the nature of the polymer matrix constituents of this novel composite film led to their longer drying time, of 72 h. Their FTIR characterization revealed the successful incorporation of amygdalin into the synthesized films, without its presence inducing any chemical modification of the constituents of the polymer matrix, their characteristic peaks being found in all the analyzed samples. Regarding the thermogravimetric analysis, it can be said that the increase in the amygdalin concentration in the films ( $\kappa$ Am1– $\kappa$ Am4) induces a shift towards higher temperatures for the  $T_{\text{onset}}$  and  $T_{\text{max}}$  values of the thermal process associated with the initiation of  $\kappa$ -carrageenan decomposition. This shift is associated with a higher thermal stability of the analyzed patches. Regarding the in vitro test, it can be said that the doped samples do not present a satisfactory efficiency in the case of *S. aureus*; a decrease in viability directly proportional to the increase in the amount of incorporated active substance was observed. In contrast, increasing the concentration of amygdalin results in a bactericidal efficiency of  $\approx 54\%$  in the case of samples  $\kappa$ Am2,  $\kappa$ Am3, and  $\kappa$ Am4 tested on *E. coli* culture. In the case of these three samples, a plateau effect in bactericidal efficiency was observed, possibly indicating that increasing the concentration of amygdalin does not significantly increase the bactericidal efficacy of the film. It was also observed that the lowest concentration of amygdalin (sample  $\kappa$ Am1) presents a much lower bactericidal efficiency even than the control sample ( $\kappa$ Am0), which indicates that, at this concentration, the active substance cannot induce an antibacterial response. These results suggest that amygdalin may serve as a carbon or energy source for *E. coli* at low concentrations. In contrast, for *S. aureus*, it can be said that HPMC from the film matrix becomes a carbon source with a negative impact on the bactericidal activity of the tested films, this component being a limitation of their use in the field of wound dressings. In conclusion, it can be said that the polymer matrix of these novel ternary biopolymer films, in combination with amygdalin, could be a promising candidate for the development of wound dressings aimed at treating infections caused by *E. coli*. However, for their practical implementation, more extensive research is needed to determine the influence of storage conditions, packaging method, shelf life, etc., to ensure that the active substance in the film can exert the desired therapeutic effect at the time of use. Future development plans will focus on scale-up production strategies, long-term stability studies, regulatory pathway assessment, and broadening the antimicrobial spectrum, thereby facilitating the translation of these ternary biopolymer films from laboratory research to clinical application.

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## Abbreviations

The following abbreviations are used in this manuscript:

|      |                                         |
|------|-----------------------------------------|
| FTIR | Fourier Transform Infrared Spectroscopy |
| TG   | Thermogravimetric analysis              |
| DTG  | Derivative thermogravimetric analysis   |
| PHB  | poly(3-hydroxybutyrate)                 |
| KC   | κ-carrageenan                           |
| HPMC | hydroxypropyl methylcellulose           |
| GEL  | Gelatine                                |
| GLY  | Glycerol                                |
| AMY  | Amygdalin                               |
| TTC  | 2,3,5-triphenyltetrazolium chloride     |
| TSA  | Tryptic Soy Agar                        |
| TSB  | Tryptic Soy Broth                       |

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